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Original Communication

In Vitro Assessment of a Pediatric Multivitamin Gummy Supplement: Effects on Probiotic Growth, Cellular Metabolism, and Antioxidant Activity

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Abstract

Background: Adequate vitamin intake during childhood is crucial for growth, immune development, and metabolic balance. Despite this, vitamin deficiencies remain common, partly due to poor adherence linked to taste and administration difficulties. Multivitamin gummies offer a palatable alternative, but their biological effects on cells and probiotic activity are not fully understood. This study aimed to evaluate the *in vitro* impact of a pediatric multivitamin gummy supplement on mammalian cells and selected probiotic strains. **Methods:** Cytotoxicity, metabolic activity, and cell morphology were assessed for primary dermal fibroblast normal, neonatal (HDFn) and human colorectal adenocarcinoma (CaCo2) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Antioxidant activity was measured by intracellular reactive oxygen species detection under hydrogen peroxide-induced oxidative stress. Probiotic growth effects were evaluated for *Lactobacillus rhamnosus* and *Saccharomyces boulardii* by enumerating colony forming unit (CFU/mL) using the serial dilution and agar plating method after incubation. **Results:** No cytotoxicity was observed in HDFn or CaCo2 cells; metabolic activity remained above the 70% viability threshold across all concentrations (1–10 mg/mL). In some doses, the supplement significantly enhanced metabolic activity compared to the control ($p < 0.05$), with a maximum increase of approximately 113% for HDFn cells at 1 mg/mL. The supplement demonstrated significant antioxidant protection by reducing reactive oxygen species (ROS) accumulation compared to Hydrogen peroxide (H_2O_2)-only treatment ($p < 0.0001$), lowering fluorescence level from over 470% in the stressed group to approximately 300% in HDFn and from 250% to below 190% in CaCo2 cells. Furthermore, it significantly increased the growth of *L. rhamnosus* ($p < 0.05$), reaching nearly 130% of the control value at the 10 mg/mL dose, while for *S. boulardii*, the observed growth increase was not statistically significant ($p > 0.05$). **Conclusions:** This study shows that the pediatric multivitamin gummy supplement is safe for mammalian cells, provides antioxidant defense under stress, and supports probiotic proliferation *in vitro*. These findings suggest that multicomponent vitamin formulations may offer synergistic biological benefits beyond basic nutrition.

Keywords: probiotic; vitamin supplement; antioxidant activity

1. Introduction

Vitamins are an important part of our daily diet. A deficiency can lead to many disorders in the body, affecting the functioning of individual organs and systems. Deficiencies can also lead to mental disorders, including depression [1,2,3]. Adequate intake of essential vitamins and micronutrients during childhood is fundamental for supporting proper growth, immune system development, and overall metabolic homeostasis. Population studies have demonstrated substantial deficiencies in several nutrients, including iodine, vitamin E, calcium, iron, riboflavin, folic acid, and vitamin C [4], with more than 50% of the surveyed population exhibiting inadequate levels. To meet the challenges associated with this, the food industry is placing increasing emphasis on food fortification. The use of additional supplementation in food makes it possible to reduce the incidence of diseases caused by deficiencies [5].Vitamins can also be used to protect and increase the stability of food, including fat-soluble vitamin E, which protects against undesirable changes in color and taste thanks to its antioxidant properties [6].

Although, vitamin supplementation is essential for children suffering from nutrient deficiencies, several difficulties and challenges have been identified regarding the consistent administration of vitamins by parents and caregivers. Among them, low motivation, lack of awareness, and poor adherence to vitamin supplementation, were the most disputed [7,8,9,10]. An additional obstacle is children's reluctance to take prescribed vitamins or medicines. The formulation of a supplement and its taste often play a crucial role in determining its acceptability and compliance [11]. What is more, in the context of oral supplementation, taste, palatability and ease of administration are recognized as important factors influencing adherence. For instance, literature reviews indicate that the perception of taste and ease of intake significantly affects whether patients consistently follow supplementation regimens [12].

Vitamin gummies and other processed forms of supplements, including chewing gum, plant-based gummies, etc., are nowadays becoming an attractive form of supplementation. They are increasingly competing with traditional tablets, often also in terms of bioavailability. One clinical study conducted on gummies with added vitamin D3 showed that gummies had higher vitamin bioavailability compared to tablets [13]. Similar results were presented in a study on vitamin C, where both gummies and tablets containing it had similar bioavailability [14]. In addition, the use of microencapsulation of vitamin C in gummies promotes its durability and stability [15]. Vitamins E, B12, and folic acid in gummies and tablets were also tested and offered similar bioavailability of these vitamins, while in terms of faster folic acid absorption, gummies had better properties [13,16,17]. Similar conclusions were drawn in studies on chewing gum, the use of which effectively increases the levels of water-soluble and fat-soluble vitamins in plasma [18]. A substantial body of evidence indicates that combined supplementation with multiple vitamins may produce synergistic effects that exceed the benefits of administering individual micronutrients alone. Beyond the convenience of specific formulation of supplements, however, growing scientific interest is directed toward evaluating whether such formulations can provide measurable biological benefits at the cellular and microbiological levels.

Importantly, despite the increasing popularity of gummy-based supplements in pediatric populations, there is a surprising lack of independent, peer-reviewed data addressing how these complex matrices, comprising gelling agents, sugars, and active compounds, interact with probiotics and mammalian cells. Therefore, the novelty of the present study lies not in methodological complexity, but in its practical and clinically relevant application, addressing a real-world formulation widely used by children and recommended by caregivers.

This study was designed as a preliminary, *in vitro* screening step prior to conducting more complex and ethically demanding clinical trials in pediatric populations. Establishing a biological baseline, including safety (non-cytotoxicity) and fundamental bioactivity, is a necessary prerequisite for further translational research. In this context, our findings provide the first empirical evidence that a gummy-based multivitamin delivery system maintains, and in some cases enhances, the biological activity of its components.

Furthermore, this research addresses a clinically relevant knowledge gap by evaluating the concentration-dependent effects of the supplement on cellular metabolism (primary dermal fibroblast normal, neonatal (HDFn) and human colorectal adenocarcinoma (CaCo2) models) and probiotic viability. These parameters are critical for defining safe exposure thresholds and for understanding potential interactions within the gastrointestinal environment. Such insights are directly relevant for clinicians and parents, as they reflect the real-world use of commercially available formulations rather than simplified experimental systems.

2. Materials and Methods

2.1 Probiotic Strains, Cell Line, and Culture Conditions

Commercially available, strains of *Lactobacillus rhamnosus* (Life Probiotyk One, GLOBAL PHARMA Poland) and *Saccharomyces boulardii* (Enterol, Francja), were employed to evaluate the potential beneficial effects on probiotic strains. DeMan, Rogosa, Sharpe medium (MRS) or Yeast Extract Peptone Dextrose medium (YPD) was used for the preparation of bacteria or yeast culture, respectively. All media were purchased from the BTL Company (Poland). Bacterial or yeast strains were cultivated at 37 °C or 30 °C, respectively, under aerobic conditions with shaking at 150 rpm (Biosan PST-60HL-4). Overnight cultures were subcultured (1:100 dilution) into fresh medium and grown to the early exponential phase. For colony-forming unit (CFU) determination, cultures were serially diluted in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) and plated on MRS (bacteria) or YPD (yeast) agar plates.

HDFn and CaCo2 cell lines were obtained from American Type Culture Collection (ATCC, USA). According to the supplier-provided certificate of analysis, the cells were characterized by normal morphology and cell-specific marker staining, with TE-7 positivity and Pan-CK negativity. The cells also tested negative for mycoplasma and microbial contamination. To prevent bacterial and mycoplasma contamination, cell lines were routinely maintained using Normocin and Plasmocin during every passage, which is recommended when working with cell lines. Cells were maintained in appropriate growth media: Fibroblast Basal Medium (ATCC, PCS-201-030, USA) supplemented with Fibroblast Growth Kit (ATCC, PCS-201-040, USA) for HDFn cells, and Eagle's Minimum Essential Medium (EMEM) for CaCo2 cells. All media were supplemented with 10% fetal bovine serum (FBS, Biowest, France) and 1% antibiotic–antimycotic solution (Sigma-Aldrich, USA). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Subculturing was performed using 0.25% trypsin-EDTA (PAA, Germany).

The composition of the dietary supplement used in this study (Table 1) was determined based on the manufacturer's specifications and is presented per recommended daily reference intake (RI) for 1 gummy as follows: Vitamin C – 40 mg (50% RI); Niacin – 8 mg (50% RI); Vitamin E – 6 mg (50% RI); Zinc – 5 mg (50% RI); Vitamin B6 – 0.7 mg (50% RI); Riboflavin – 0.7 mg (50% RI); Vitamin A – 400 µg (50% RI); Folic acid – 100 µg (50% RI); Iodine – 45 µg (50% RI); Selenium – 27.5 µg (50% RI); Biotin – 25 µg (50% RI); Vitamin D3 – 2.5 µg (50% RI); Vitamin B12 – 1.25 µg (50% RI).

Table 1. Quantitative composition and nutritional value of the pediatric multivitamin gummy supplement.

Ingredient	Amount per gummy	% Reference intake (RI)
Vitamin C	40 mg	50%
Niacin (B3)	8 mg	50%
Vitamin E	6 mg	50%
Zinc	5 mg	50%
Vitamin B6	0.7 mg	50%
Riboflavin (B2)	0.7 mg	50%
Vitamin A	400 µg	50%
Folic acid	100 µg	50%
Iodine	45 µg	50%
Selenium	27.5 µg	50%
Biotin	25 µg	50%
Vitamin D3	2.5 µg	50%
Vitamin B12	1.25 µg	50%

2.2 Effect of Vitamin Supplementation on Probiotics Growth

Bacterial and yeast precultures were incubated overnight under aerobic conditions at 37 °C and 30 °C, respectively, and subsequently diluted to an initial concentration of approximately 1×10^6 cells/mL. Aliquots (100 µL) of the supplement were mixed with 100 µL of the bacterial suspension and incubated for a further 24 h with shaking. The final concentrations of the supplement, calculated as the total mass of the gummy formulation (including active ingredients and excipients) per volume of medium, were 10, 5, and 1 mg/mL. After incubation, control and treated samples were serially diluted and plated onto agar plates. Colony forming unit (CFU) were enumerated and expressed as CFU/mL. All experiments were performed in triplicate and were conducted in a laminar flow hood (Thermo Scientific MSC Advantage).

2.3 Biological Properties of Vitamin Supplement

Metabolic activity of human fibroblasts was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [19], with modifications. Briefly, cells were seeded onto the 96-well plate at a density 4×10^4 cell/well and allowed to attach for 24 h. Subsequently, the medium was replaced with 200 µL of fresh medium containing different concentrations of the supplements. After 24 h incubation, the medium was removed and replaced with 200 µL of MTT (Sigma-Aldrich) solution (0.5 mg/mL in culture medium). After 4 h of additional incubation, the supernatant was discarded and the formazan crystals were dissolved with 100 µL of DMSO. Absorbance was measured at 570 nm using a microplate reader (Tecan Infinite Pro200, Thermo Fisher). Results were expressed as the percentage of control values (non-treated cells).

For morphology analysis, HDFn and CaCo2 cells were seeded in 24-well plates at a density 5×10^5 cell/well and incubated for 24 h. Cells were then treated with supplement at the same concentration as in the MTT assay. Cell morphology was evaluated using bright-field microscopy (Inverted Microscope, Olympus IX).

2.4 Antioxidant Activity of Supplement

HDFn and CaCo2 cells were seeded in black 96-well plates at a density of 4×10^4 in cell/well and incubated for 24 h to allow attachment. Growth medium was removed and the cells were washed with PBS to remove any non-adherent and dead cells. Then, 100 µL of 10 µM DCFH-DA working solution was added to each well, and the cells were placed in the incubator for 1 h. After this period, the cells were washed twice with PBS to remove excess probe. Next, 100 µL of the supplement, culture medium (negative control), or 100 mM Hydrogen peroxide (H_2O_2) (positive control) was, followed by 24 h incubation. Fluorescence was measured at an excitation/emission wavelength of 495/535 nm using a microplate reader (Tecan Infinite Pro200, Thermo Fisher). Results were expressed as relative fluorescence compared to controls.

2.5 Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical analysis was performed using one-way Analysis of Variance (ANOVA), followed by Dunnett's post hoc test or Tukey's multiple comparisons for MTT or ROS generation analysis, respectively. Analyses were conducted using GraphPad Prism version 10 (Boston, Massachusetts USA). A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Positive Effect on the Probiotics Growth

Among the most documented probiotic strains, *Lactobacillus rhamnosus* and *Saccharomyces boulardii* have been associated with several health-promoting properties [20]. Indeed, there are reports on the micronutrient supplementation which influences the composition and diet-

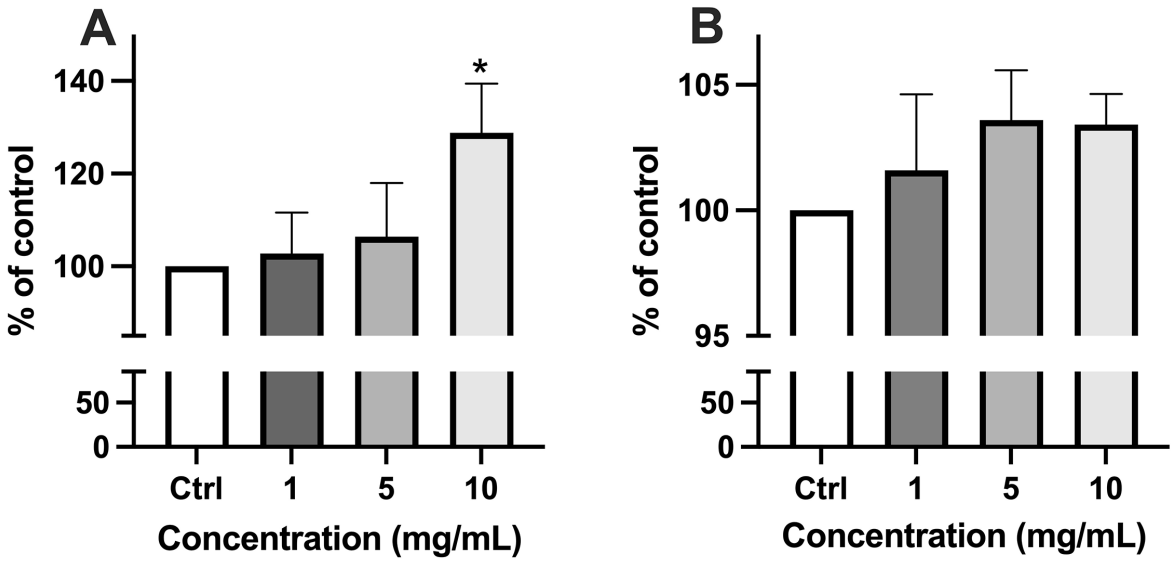

Fig. 1. Effect of vitamin supplementation on the probiotic growth expressed as percentage of control based on colony forming unit. Treatment with the vitamin supplement resulted in an increase in viable cell counts for *Lactobacillus rhamnosus* (A) or *Saccharomyces boulardii* (B) compared to untreated control. Concentrations (1, 5, 10 mg/mL) represent the total weight of the gummy supplement dissolved in the medium. Data are presented as mean ± SD from three independent experiments. Statistical significance: $p < 0.05$ versus control. SD, standard deviation. The significant differences are denoted at $* p \leq 0.05$.

originating function of the gut microbiome in healthy adults or treated mice [21,22]. However, much existing evidence is inconsistent and often involves combinations of vitamins with other interventions (e.g., probiotics, omega-3 fatty acids), which makes it difficult to attribute the effect solely to the vitamin itself on the gut microbiota. On the other hand, most *in vitro* works use vitamins only in the context of interaction with pathogens or host cells (e.g., epithelial cells), or study general probiotic properties (acid/bile tolerance, adhesion) — not vitamin-driven growth or expansion of probiotic strains. Therefore, the effect of the vitamin supplement (MULTI Zdrowe Żelki) proposed by Pexon Poland on the proliferation of probiotic strains was evaluated. We found that treatment with the vitamin supplement increases the cell number of *L. rhamnosus*, and *S. boulardii* (Fig. 1). It should be emphasized that the strongest and statistically significant positive effect on bacterial population is observed at the highest vitamin concentration. For yeasts, slight differences between groups can be observed; however, these changes were not statistically significant ($p > 0.05$). The gut microbiome plays essential roles in host physiology, including immune modulation, protection against pathogens, detoxification, regulation of bowel function, and the synthesis, absorption, and metabolism of nutrients. Emerging evidence suggests that vitamins, particularly B-group vitamins, may act as potential modulators of the gut microbiome and play an important role in shaping its diversity and richness [23,24]. Accordingly, their supplementation in cases of deficiency or following antibiotic therapy—when the gut microbiota is altered, and vitamins cannot be synthesized by the host microbiome—may have significant beneficial effects.

3.2 Vitamin Supplementation Exerts Positive Effects on Mammalian Cells

The principle of human supplementation must always rely on ensuring the absence of deleterious effects on organs and cells. Accordingly, the impact of the vitamin supplement on human fibroblasts and intestinal cells was evaluated, regarding the cytotoxicity effect. Human primary fibroblasts play an important role in maintaining the structural integrity of connective tissue and in the synthesis of extracellular matrix proteins [25]. The CaCo2 cells are widely used for mimicking the small intestine in the gastrointestinal (GI) system [26]. Cellular metabolic activity was used as a primary parameter to assess cytotoxicity, reflecting the overall functional status of the cells. It was tested using the MTT assay, which measures cell mitochondrial activity through the NAD(P)H-dependent cellular oxidoreductase enzyme [27]. The MTT assay consists of monitoring any reduction in the ability of cells to degrade MTT into formazans in the presence of a potential perturbator (i.e., drug). For these tests, confluent HDFn and CaCo2 cells were incubated in the presence of different concentrations of vitamin (1–10 mg/mL). After 24 h of incubation, the cell metabolic activity was assessed based on the conversion of MTT into formazans that were quantified by spectrophotometry. Cells with metabolic activity below 70% relative to control are considered cytotoxic, following established MTT assay interpretation [28]. Within the tested concentration range, no cytotoxic effect was ob-

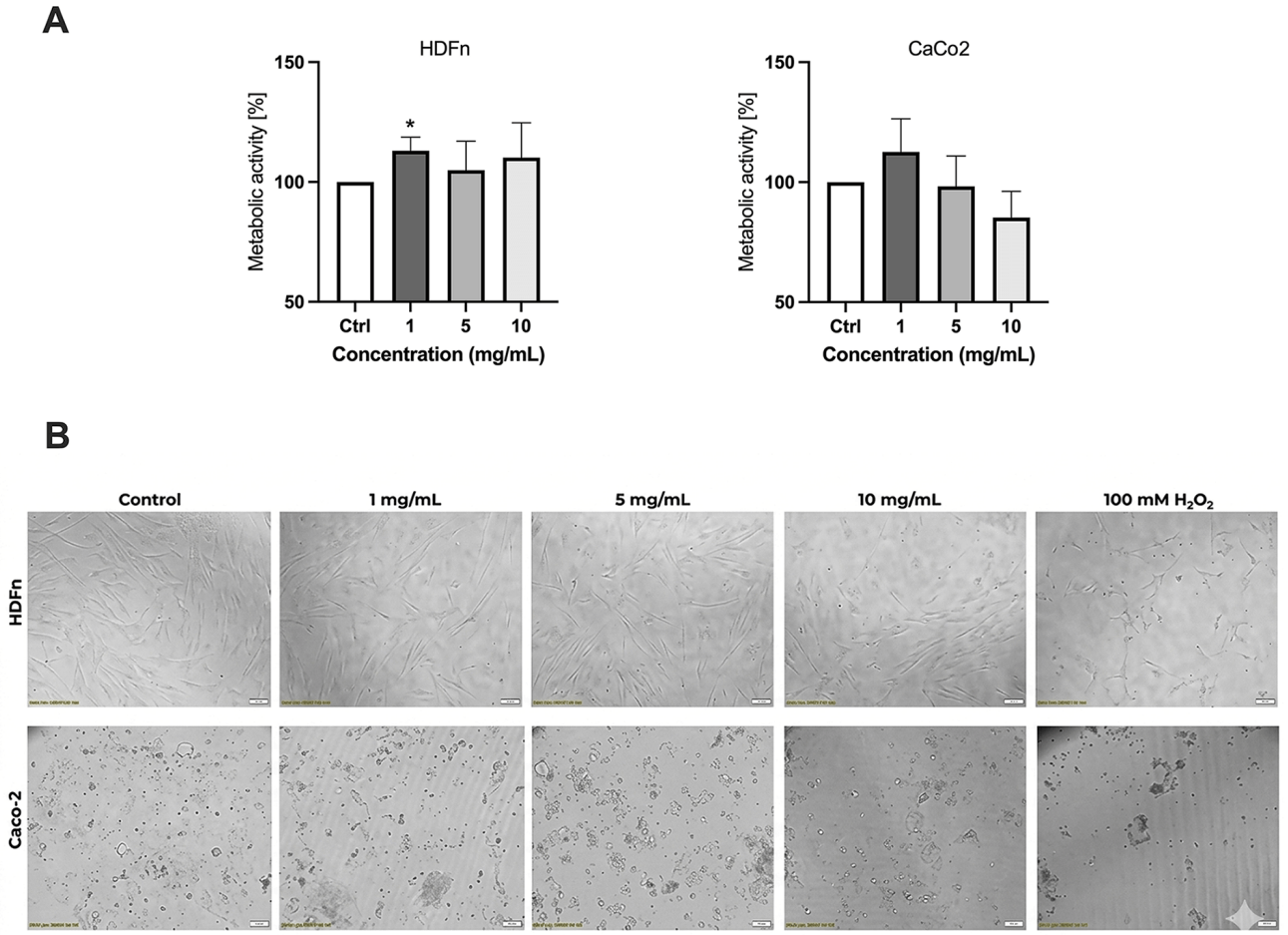

Fig. 2. Cell response to vitamin supplement treatment. (A) Percentage of metabolic activity of cells after 24 h of exposure to different concentrations of vitamin supplement. Cells were treated with three increasing concentrations of the supplement, and after 24 h of exposure metabolic activity was assessed. Data analysis was carried out by one-way Analysis of Variance (ANOVA) with Dunnett's multicomparison test. Concentrations (1, 5, 10 mg/mL) represent the total weight of the gummy supplement dissolved in the medium. Data are presented as mean ± SD, statistical significance: $p < 0.05$ versus control. No statistical significance was found for the human colorectal adenocarcinoma (CaCo2) cell line. (B) Morphology of primary dermal fibroblast normal, neonatal (HDFn) and CaCo2 cells after exposure to different concentrations of supplement. Cells were treated with supplement (1, 5, 10 mg/mL) and after 24 h were imaged under the microscope. Scale bar, 100 µm. The significant differences denoted at $* p \leq 0.05$.

served for HDFn and CaCo2 cells (Fig. 2A). On the contrary, the metabolic activity of the cells increased for some of the tested concentrations. This suggests that the synergistic effects of the vitamin compounds may enhance cell proliferation and potentially support intestinal tissue development. Indeed, some studies have reported the synergistic nutraceutical combinations that stimulate the proliferation of human progenitor cells. However, it is also important to consider that such effects are strongly concentration-dependent [29,30]. To confirm no cytotoxicity after supplement administration, the cell morphology was analyzed (Fig. 2B). Cells grown in the presence of different concentrations of the tested supplement exhibited normal morphology, characteristic of fibroblasts and epithelial cells. They adhered and proliferated in a manner comparable to untreated cells. In contrast, exposure to the stress-stimuli factor, hydrogen peroxide, resulted in a marked decrease in cell density and pronounced morphological alterations, suggesting cell death.

3.3 Vitamin Supplementation Protects Cells Against Oxidative Stress

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, generated in a cellular response to bacterial invasion, xenobiotics, heavy metal, etc. Oxidative stress occurs when there is an imbalance between oxidants and the cell's antioxidant defenses, which can further damage lipids, proteins, and DNA, impairing cell function. Therefore, assessing the antioxidant capacity of dietary supplements helps understand their potential to protect cell from oxidative damage. To further evaluate the effects of supplement on oxidative stress, we com-

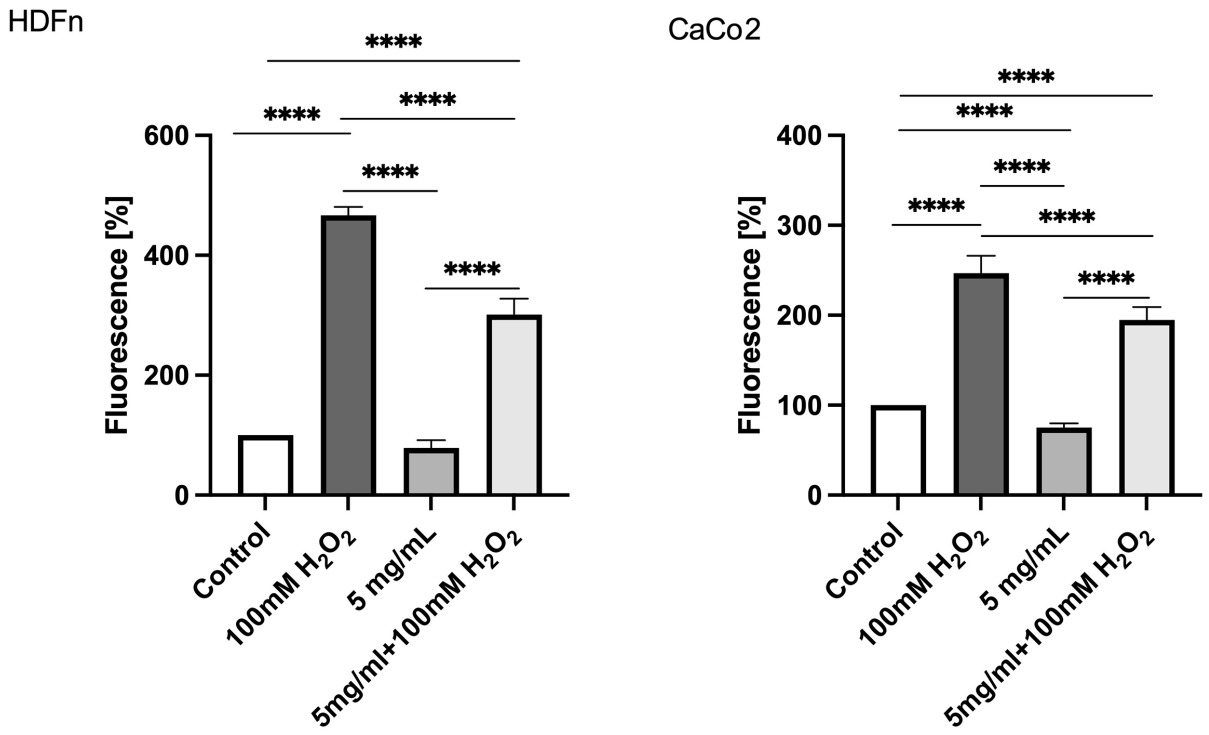

Fig. 3. Cellular antioxidant activity of vitamin supplement in CaCo2 and HDFn cells. Cells, incubated in black 96-well culture plates, were treated with 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) (10 µM) and then incubated with 5 mg/mL vitamin or vitamin with H_2O_2 (100 mM) for 24 h. Intracellular ROS were measured using a microplate reader and are expressed as the percent of the fluorescence compared to control. Values in control samples were arbitrarily set at 100%, with values in treated samples plotted as a percentage of the control. Concentrations (5 mg/mL) represent the total weight of the gummy supplement dissolved in the medium. Data were expressed as the mean values with standard deviations; **** $p < 0.0001$ with the one-way ANOVA, Tukey's multiple comparisons test. ROS, reactive oxygen species.

pared the level of ROS generation during the stress response (stimuli with hydrogen peroxide). As presented in Fig. 3, the production of ROS was markedly increased in HDFn and CaCo2 cells exposed to H_2O_2. While the accumulation of ROS in the cells in the presence of the vitamin and hydrogen peroxide simultaneously, was significantly reduced compared to the H_2O_2-only treatment. We found that the vitamin supplement may have cytoprotective effects on oxidative stress in HDFn and CaCo2 cells. A similar effect was observed for the keratinocytes after combined treatment with several natural antioxidant compounds. Indeed, when the components were administered in combination at the highest concentrations used, they markedly and significantly reduced ROS production [31]. All these results together support the hypothesis that the simultaneous administration of components with antioxidant activity may result in improved distribution and enhanced antioxidant effects.

4. Conclusions

This study shows that the tested vitamin supplement promotes probiotic growth, is non-cytotoxic to HDFn and CaCo2 cells, and exhibits antioxidant activity. It enhanced the proliferation of *Lactobacillus rhamnosus* and *Saccharomyces boulardii*, particularly at higher concentrations, while supporting cellular metabolic activity. Overall, the findings suggest that combined vitamins may improve probiotic viability, ensure cellular safety, and protect against oxidative stress, although further *in vivo* studies are needed.

Availability of Data and Materials

All data reported in this paper will also be shared by the lead contact upon request.

Author Contributions

MKL designed the research study. MKL and JG performed the research. RP provided material for the study. MKL and JG wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflicts of Interest

All authors declare no conflicts of interest. Despite testing the product from Pexon, this relationship did not influence the data interpretation or the conclusions drawn.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT-3.5 in order to check spell and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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